Induction of Hypertrophy in Human Cartilage Endplate Cells Promotes Angiogenesis and Catabolism in the Intervertebral Disc

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Biomedical Engineering in the undergraduate colleges of The Ohio State University

By

Taylor Yeater

The Ohio State University

May 2017

Project Advisor: Devina Purmessur, Department of Biomedical Engineering

Abstract

Low back pain is second only to cancer in terms of socioeconomic burden in the U.S., but current treatments are highly invasive and fail to target the underlying cellular mechanisms of intervertebral disc (IVD) degeneration. In degeneration, the cartilaginous end plate (CEP) becomes calcified, however the mechanisms are not well understood. In diseases such as osteoarthritis, a proposed mechanism is the recapitulation of developmental processes and we suggest that in degeneration, CEP cells undergo hypertrophic differentiation similar to endochondral ossification. The aim of this study was to determine if CEP cells can undergo hypertrophic differentiation, leading to angiogenesis. Human CEP cells (hCEPs) were isolated from autopsy and pellet cultured. For 21 days, pellets were cultured in either chondrogenic or hypertrophic (10% FBS or CHIR99021, a wnt agonist) media in 5% oxygen, after which samples were used for viability, histology, qRT-PCR, DMMB proteoglycan assay, and generation of conditioned media (CM). Soluble factors from CM generated after 21 days was pooled, and a HUVEC tubular formation assay ran. Tubular length was assessed using Image J plug in, Angiogensis Analyzer. No significant decreases in viability were seen in the chondrogenic control group. QRT-PCR showed a decrease of chondrogenic marker, COL2, in both hypertrophy groups compared to the chondrogenic group. Hypertrophic markers MMP13 and IHH displayed increases in the hypertrophy groups. Increases were also seen in the angiogenic and pain related markers, VEGF-A, TAC1, and NGF in the hypertrophy groups. DMMB assay and histological results show decreases in proteoglycans in the hypertrophy groups. HUVEC tubular formation assay showed that both hypertrophy groups increased the total length of tubules. This study sought to determine the underlying cellular mechanisms of low back pain and

suggests that human CEPs have the ability to undergo hypertrophic differentiation similar to articular chondrocytes during OA, with subsequent angiogenesis and neoinnervation, and may be implicated in the process of IVD degeneration.

Acknowledgement

I am appreciative of the support, guidance, and technical backing of my research mentor, Dr. Devina Purmessur. I would like to thank members of the Spinal Therapeutics Lab for providing a collaborative and helpful work environment, which allowed me to complete this project in an efficient manner: Pavel Sul, Andrew Piscioneri, Matthew Wiet, Brian King, Katie Lakstins, and Kelly Thompson. I would also like to thank Dr. Keith Gooch for sitting on my defense committee.

The work completed in the project would not have been possible without the financial support of the Department of Biomedical Engineering in the College of Engineering at The Ohio State University**.** I am additionally grateful to The Ohio State University's College of Engineering for recognizing the potential of the proposed project and awarding me a scholarship to complete this thesis.

Contents

List of Figures

List of Tables

Background

Significance and problem statement

Low back pain (LBP) is one of the most prevalent sources of socioeconomic burden, comparable to heart disease or diabetes (Maetzel & Li, 2002), and intervertebral disc (IVD) degeneration is implicated in the progression of chronic LBP (de Schepper et al., 2010; Freemont, 2009). It has been estimated that 7.1 million adults have activity limitation (Lawrence, 2008) and 70-85% of the population will experience LBP at least once during their lifetime (Andersson, 1999). Current interventions for acute or chronic LBP do not improve the economic burden, and only modestly improve clinical outcome (Maetzel & Li, 2002). Additionally, common interventions such as analgesics and physiotherapy do not address the underlying pathophysiology, but just treat the symptomatic pain. When this fails, highly invasive surgeries such as lumbar fusion are performed. This can lead to immobility and adjacent disc disease (Mannion et al., 2014). Furthermore, current treatments do not consider the cartilage endplate (CEP) – IVD as an organ system. Despite discogenic LBP being so widespread, effective and minimally invasive therapeutics based on the cellular changes in the IVD have yet to be developed.

Intervertebral disc structure and function

The intervertebral disc is comprised of three main components: the nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilage endplate (CEP). The proteoglycan-rich NP imbibes fluid and serves to transmit compressive load throughout the spine, while the lamellar structure of collagen I in the AF transmits the tensile load of the spine. The CEP

contains the main structure of the disc cranially and caudally, and is the main nutritional pathway for the avascular IVD. These structures are shown in Figure 1 (Smith, Nerurkar, Choi, Harfe, & Elliott, 2011). The IVD is normally avascular, so cells rely on diffusion for nutrients. It has been shown that lack of oxygen or glucose will cause NP cells to become quiescent or die, respectively. Because of this, the IVD has a limited ability to repair itself from metabolic or mechanical injury (Adams & Roughley, 2006).

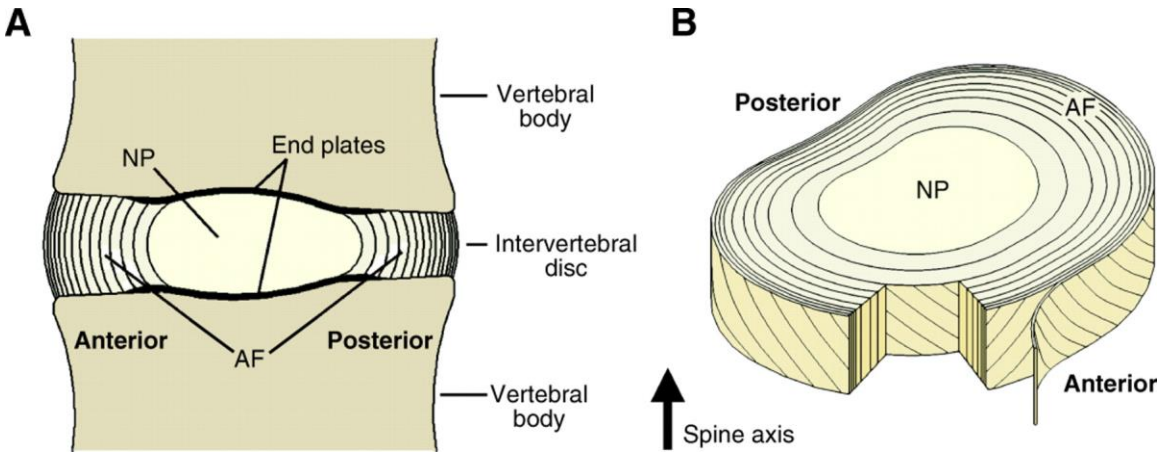

Figure 1: Structure of intervertebral disc. Nucleus pulposus (NP), annulus fibrosis (AF) and cartilage endplate (CEP) are shown

Intervertebral disc in degeneration

During the progression of IVD degeneration, there is a shift in matrix remodeling with a net loss in extracellular matrix components. The degradation of neural inhibitory proteoglycans, such as aggrecan, permit neoinnervation into the IVD. It has been suggested that it is the nociceptive fibers, which follow neovascularization into the disc, are the source of LBP (Freemont, 2009).

In disease, the CEP becomes calcified; it has been shown that degenerative CEP permeability decreases 50-60%, further limiting the transport of nutrients into the disc as well as altering disc mechanics (DeLucca et al., 2016). Other structural changes to the CEP, including osteophyte formation and hypertrophy, have been deemed precursors to IVD degeneration (Adams & Roughley, 2006; Liu et al., 2016). Structural damage to the CEP and IVD has been suggested as a predisposing disease model for bone marrow lesions visualized as Modic changes on magnetic resonance imaging, which display high specificity for discogenic LBP (Dudli, Fields, Samartzis, Karppinen, & Lotz, 2016).

Related Processes

Related musculoskeletal diseases, such as osteoarthritis (OA), display similar pathology to IVD disease, and may provide insight into potential disease mechanisms. Both OA and IVD disease display increased matrix breakdown, inflammatory response, and neurovascular ingrowth (Freemont, 2009; Goldring, 2012). A proposed mechanism associated with pathogenesis of diseases such as OA is the recapitulation of developmental processes, and it has been shown that in diseases such as cancer, pulmonary fibrosis, and OA that pathophysiology progresses via similar mechanisms seen in development (Borczuk et al., 2003; Goldring, 2012; Selman, Pardo, & Kaminski, 2008).

One such mechanism is hypertrophic differentiation and subsequent ossification of chondrocytes. During long bone formation, chondrocytes undergo hypertrophic differentiation that leads to matrix breakdown, chondrocyte apoptosis, and vascular ingrowth (Nishimura et al., 2012). A similar process is seen in OA chondrocytes. Pesesse *et al* showed that OA chondrocytes could undergo hypertrophic differentiation, as indicated

by gene expression and cellular morphology. Additionally, they showed that hypertrophic differentiation of OA chondrocytes could promote angiogenesis (Pesesse et al., 2013). We h that, in degeneration, the CEP becomes calcified via hypertrophic differentiation similar to OA and endochondral ossification, promoting angiogenesis and matrix catabolism in the IVD.

A hypothetical model of our aims is shown below in Figure 2. The top represents the healthy CEP, which contains intact matrix molecules such as collagen type II and proteoglycans such as Aggrecan. The CEP cells here are healthy and similar to chondrocytes. Following the arrow of degeneration to below the image, the CEP matrix degrades and becomes calcified. CEP cells become hypertrophic and neovascularization and innervation into the disc occur. We hypothesize that through therapeutic interventions targeting hypertrophic differentiation, the CEP could return to a normal, healthy environment.

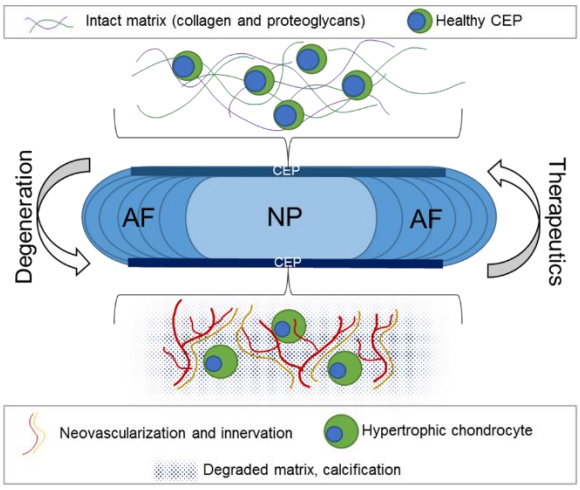

Figure 2: Hypothetical model

Specific aims and hypotheses

Aim 1: Determine the effects of hypertrophic differentiation on CEP cells using two known inducers of hypertrophy. *We hypothesize that both inducers of hypertrophy will cause an upregulation of hypertrophic, neurogenic, and pain markers along with a decrease in proteoglycan content.*

Aim 2: Determine angiogenic potential of hypertrophic CEP cells by using a functional assay of angiogenesis. *We hypothesize that conditioned media from the hypertrophic groups will cause an increase in total tubular length of HUVECs, indicating greater angiogenic potential.*

Materials and Methods

Ethics statement

Human autopsy specimens were obtained through the comprehensive human tissue network classified by the institutional review board as exempt research.

Primary cell isolation and expansion

Cartilage endplate cells were isolated from six human cadaveric spines. Ages of subjects ranged from 43 to 58; two subjects were male and four female. First, individual IVDs were isolated from each spine. The vertebral bodies were then scraped to remove the cartilage endplate tissue. Tissue was submerged in 15 ml disc cell digestion medium (see recipe below) containing 0.03 g protease and placed in a shaker at 37 degrees for 60 minutes. Media was replaced with 15 ml digestion media containing 0.0018 g collagenase II and

replaced on the shaker with the same settings overnight. After straining through a 70-micrometer cell strainer to remove digested tissue and debris, cells were expanded in plastic culture flasks in disc cell complete medium (see recipe below).

Disc cell digestion medium: 4.5 g/L glucose DMEM, 100 U/ml penicillin-100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B

Disc cell complete medium: 4.5 g/L glucose DMEM, 100 U/ml penicillin-100 µg/ml streptomycin, 0.25 µg/ml amphotericin B**,** 50 µg/ml ascorbic acid, 10% FBS

Pellet culture

CEP cells (p. 3-4) were expanded to ~80% confluence and 1 x 10^6 cells were centrifuged at 400 rcf for 5 minutes to create a cell pellet. For 21 days, pellets were cultured in either chondrogenic (positive control), 10% FBS, or Wnt Agonist (CHIR99021) media in 5% O_2, 5% CO_2, and 37°C with media changes every 3-4 days. 10% FBS medium was chosen because it has been shown to induce hypertrophic differentiation in OA chondrocytes (Pesesse et al., 2013), as was wnt agonist (Enochson, Stenberg, Brittberg, & Lindahl, 2014).

Chondrogenic medium 4.5 g/L glucose DMEM, 100 U/ml penicillin-100 µg/ml streptomycin, ITS (Insulin – 1 g/L; Tranferrin - 0.55 g/L ; Selenium- 0.0006 g/L), 20 ug/ml proline, 50 µg/ml ascorbic acid, 2 mM glutamine, 1mM sodium pyruvate, 10^{-7} M dexamethasone, and 10 ng/ml TGF-β3 (added just before media change).

10% FBS medium: 4.5 g/L glucose DMEM, 100 U/ml penicillin-100 mg/ml streptomycin, 10mM HEPES, 20 µg/ml proline, 50 µg/ml ascorbic acid, 2 mM glutamine, and 10% FBS.

Basal/Wnt agonist medium: of 4.5 g/L glucose DMEM, 100 U/ml penicillin-100 µg/ml streptomycin, 10mM HEPES, ITS (Insulin – 1 g/L; Tranferrin - 0.55 g/L; Selenium- 0.0006 g/L), 20 µg/ml proline, 50 µg/ml ascorbic acid, 2 mM glutamine (basal media), and 0.5 uM CHIR-99021 (added just before media change to create wnt agonist media).

Viability

To determine whether culture conditions were cytotoxic after 21 days, a Calcein AM/ Ethidium homodimer-1viability test was performed. Pellets in 10% FBS were first rinsed in phosphate buffered saline solution to dilute serum esterase activity. Pellets were incubated for 20 minutes in serum free media with a final concentration of 2 µM calcein and 1 µM ethidium**.** A 4x fluorescent image of each pellet was taken to qualitatively determine relative ratio of live cells to dead cells.

Generation of conditioned media

Conditioned media from three (N=3) of the 21 day pellets was generated by serum starving samples for 24 hours in basal medium and pooling media. Soluble factors were isolated from the media by centrifuging in Pall spin filters (MAP003C37) at 3000 rcf for 45 minutes**,** then re-suspended in an equal volume of Medium 200 phenol red free

supplemented with 100 U/ml penicillin-100 µg/ml streptomycin to be used in the functional assay of angiogenesis.

Histology

After 21 days in culture, pellets (N=3) used to generate conditioned media were then kept in -80°C until fixing. Pellets were fixed in 10% buffered formalin for 24 hours, dehydrated, then imbedded in paraffin and sliced into 4 µm sections. To determine matrix production, the sections were deparaffinized and stained using picrosirius red (collagen) and alcian blue (proteoglycans) (Walter et al., 2011).

Matrix (Proteoglycan) production

Pellets (N=5) were kept in -80°C before biochemical analysis of proteoglycan content. First, samples were digested in papain. 200 µl was added to each sample and incubated in 60°C overnight. After digest, samples were vortexed and used for dimethylmethylene blue (DMMB) assay to assess glycosaminoglycan (GAG) content (Luo et al., 2016). A standard curve was generated using known concentrations of GAG to determine GAG content in pellets. To determine DNA concentration, Hoescht 33258 assay was used per manufacturer's instructions. GAG concentration was normalized to DNA concentration.

Gene expression

To determine relative gene expression, qRT-PCR was performed on pellets from each group. PureLink RNA Mini Kit (Invitrogen, 12183018A) was used to isolate RNA. Samples from the first three subjects were lysed using 600 µl lysis buffer included with the RNA purification kit. 500 µl Trizol Reagent (Ambion, 15596026) was used to lyse

remaining samples due to poor pellet digestion with lysis buffer. RNA content was quantified using Nanodrop 2000, and was stored in -80°C until complementary deoxyribose nucleic acid (cDNA) conversion. Samples were converted to cDNA using qScript XLT cDNA SuperMix (Quanta Biosciences 95161), diluted to a final concentration of 10 ng/reaction. Samples were stored in -20°C until qRT-PCR. Finally, qRT-PCR was done in duplicate using Taqman Master Mix and primers (Table 1). Gene expression was determined using the ΔΔCT method relative to the housekeeping gene 18s (Purmessur et al., 2011).

Table 1: genes tested and role in IVD

Target gene	Role
18s	Housekeeping gene
COL2A1	Matrix marker; indicates chondrogenic phenotype
SOX9	Matrix marker; indicates chondrogenic phenotype
IHH	Hypertrophic marker
MMP13	Catabolic marker; indicates hypertrophic phenotype
RUNX2	Hypertrophic marker
VEGFA	Angiogenic marker
NGF	Neurogenic marker
TAC1	Substance P; indicates pain

Tubular formation assay

A functional assay of angiogenesis utilizes the ability of Human Umbilical Vein Endothelial Cells (HUVEC) to spontaneously form tubular microcapillary networks on extracellular matrix, such as Geltrex when pro-angiogenic factors are present (Donovan, Brown, Bishop, & Lewis, 2001). HUVECs were expanded to 80% confluence. 100 µl of Geltrex was pipetted into each well of a 24-well plate, keeping the Geltrex on ice. Plates were then placed in 37°C for 30 minutes to allow Geltrex to gel. HUVECs were trypsinized

and $8.5x10^4$ cells/well in 400 µl media were seeded in each well (N=4 wells/condition). Positive control was Medium 200 phenol red free + 100 U/ml penicillin-100 µg/ml streptomycin + low serum growth supplement. Negative control was Medium 200 phenol red free + 100 U/ml penicillin-100 µg/ml streptomycin.

Statistical Analysis

GraphPad Prism version 6 software was used to determine statistical significance. Sample size for each test ranged from N=4 to N=6. Kruskal Wallis nonparametric test was used with Dunn's post-hoc analysis**.** Results were significant if $P < 0.05$.

Results

Long-term culture of hCEPs does not affect cell survival in control pellet culture

To ensure cells were remaining viable throughout the 21-day study, a Calcein AM/ Ethidium homodimer-1 assay was performed. As can be seen qualitatively in Fig. 3, the chondrogenic (positive) control group showed the highest cell survival, as indicated by green (live cell) areas. In contrast, the 10% FBS (hypertrophic) group exhibited more cell death, as indicated by the red (dead) areas. The wnt agonist (hypertrophic) group displayed viability between the chondrogenic and the 10% FBS groups.

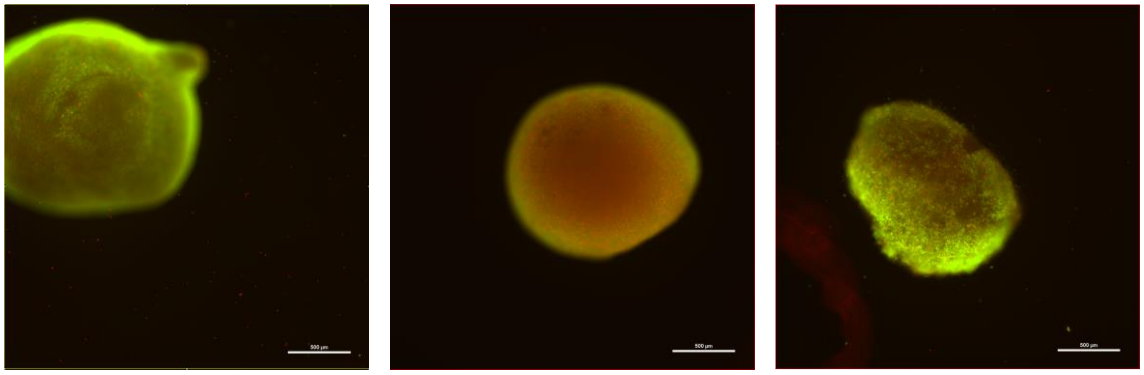

Figure 3: Representative images of live/dead assay

Hypertrophic differentiation of hCEPs promotes catabolism

Images of pellets were taken after 21 days in culture to compare relative size of pellets. As can be seen in Figure 4, chondrogenic pellets appeared the largest. 10% FBS pellets were the smallest, and the wnt agonist group had a pellet size between the chondrogenic and 10% FBS groups

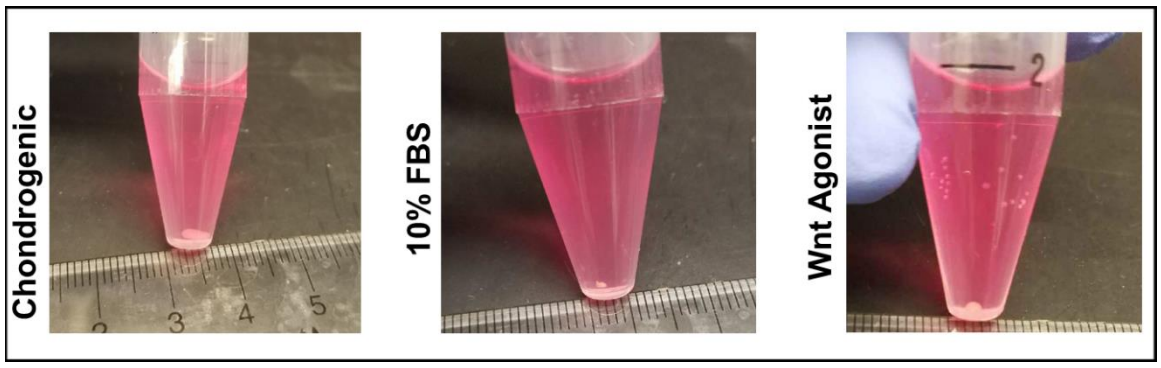

Figure 4: Representative images of pellet size

Picrosirius red and alcian blue staining was done to determine the matrix accumulation and visualize the distribution of collagen (red) and proteoglycan (blue). As can be seen in Fig 5, the chondrogenic control group has the most proteoglycan content, as indicated by the

mostly blue appearance of the pellet. In the 10% hypertrophic group, proteoglycan presence was greatly diminished, as the pellet is mostly red. The wnt agonist pellet is also mostly comprised of collagen, but faint blue on the right edge of the pellet indicates some proteoglycan presence.

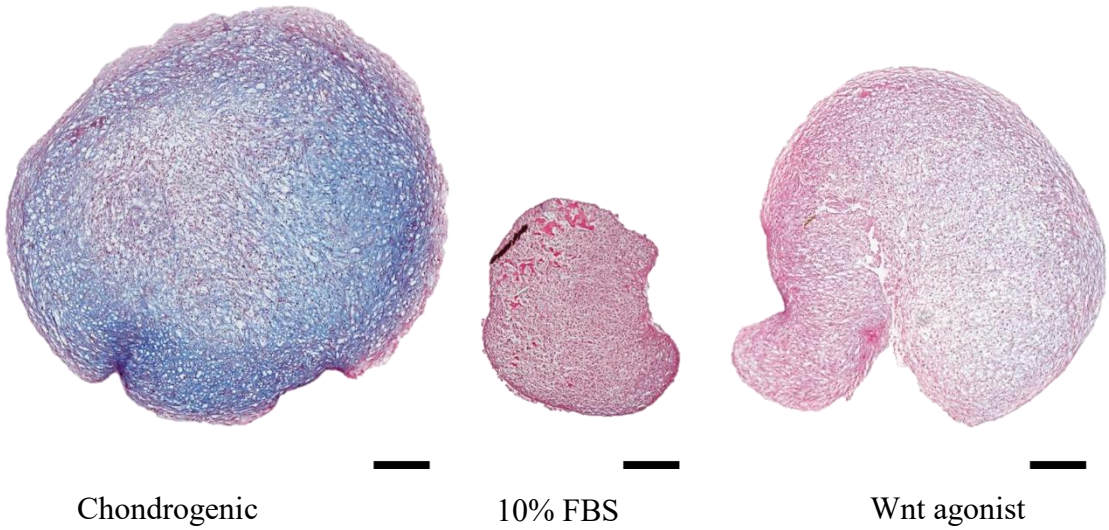

Figure 5: Representative histology images. Scale = 200 µm

Matrix accumulation was quantitatively assessed by performing a papain digest of the pellets, then determining GAG content through a DMMB assay. After normalizing to DNA content, it was seen that the chondrogenic control group accumulated the most proteoglycan. The 10% FBS hypertrophic group had significantly lower matrix accumulation. Again, the wnt agonist hypertrophic group had results between the chondrogenic and 10% FBS groups (Figure 6).

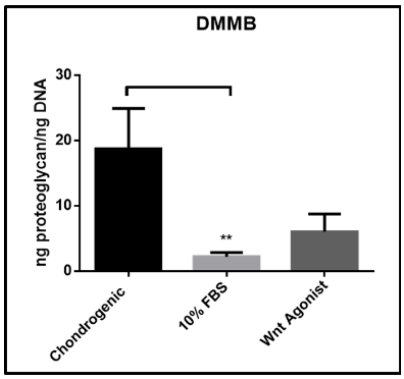

Figure 6: Results from DMMB assay. Bar indicates statistical significance (P < 0.01)

Hypertrophic phenotype of hCEPs is indicated by gene expression

After 21 days in pellet culture, cells were lysed and gene expression determined. All gene expression was normalized to the chondrogenic control. Values of fold change and P value are included in Table 2. One set of genes were matrix markers collagen type II (COL2) and SOX9. As can be seen in Figure 7 (matrix markers), both hypertrophic groups had significantly decreased COL2 production with the 10% FBS group having the largest difference. Interestingly, another matrix marker, SOX9 showed significant upregulation in the 10% FBS group. Hypertrophic markers IHh, MMP13, and Runx2 were also determined (Fig 7 (hypertrophic markers)). Upregulation, but not significant, of IHh was seen in both hypertrophic groups. The catabolic gene MMP13 showed the largest upregulation of all genes for both hypertrophic groups, and was significant in the 10% FBS group. Runx2, a transcription factor involved in IHh pathway, was upregulated in both hypertrophic groups, again with significance seen in the 10% FBS group.

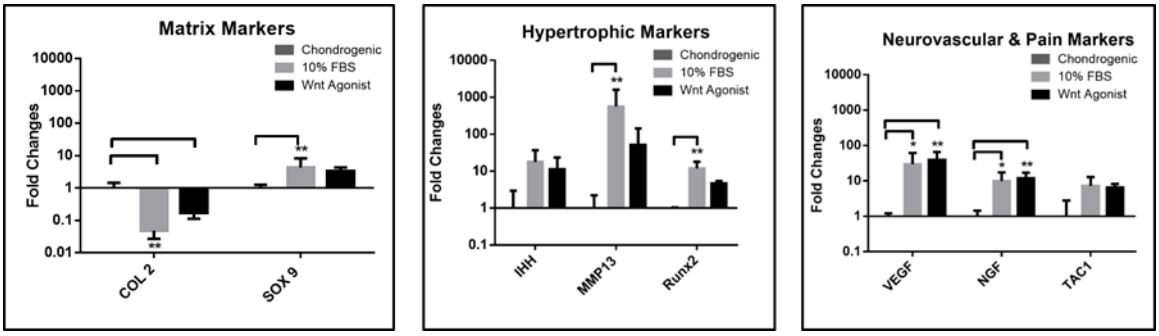

Figure 7: Gene expression of hypertrophic CEPs relative to chondrogenic control

The final group of gene expression determined were neurovascular and pain markers, which are indicative of the diseased IVD (Fig7 (neurovascular and pain markers)). The

angiogenic factor, VEGF, was significantly upregulated in both hypertrophic groups. The neurogenic factor, NGF, was also significantly upregulated in both groups. Upregulation for both genes was similar between the two hypertrophic groups, unlike the trend seen in previous results. The pain marker TAC1, or Substance P, was upregulated in both groups, but this was not significant.

Table 2: ΔΔCT values and P-values for gene expression

	10%FBS		Wnt agonist	
Gene	ddCT	P	ddCT	P
COL2	0.05	P<0.01	0.17	P<0.05
SOX9	4.27	P<0.01	3.37	P>0.05
Ihh	17.82	P>0.05	11.13	P>0.05
MMP13	564.88	P<0.01	51.54	P>0.05
Runx2	11.94	P<0.01	4.68	P>0.05
VEGF	29.69	P<0.05	39.32	P<0.01
NGF	9.90	P<0.05	11.91	P<0.01
TAC1	7.22	P>0.05	6.52	P>0.05

Hypertrophic differentiation of hCEPs promotes angiogenesis

Tubular formation of HUVEC cells was observed to determine the angiogenic potential of the CEP cells. Conditioned media from each cell pellet was applied to the HUVECs, and fluorescent images taken (Figure 8). The positive control exhibited strong tubular formation, with a few examples of thick tubules indicated by red arrows. The negative control did not form many tubules, and of those formed they were either thin or broken. The chondrogenic control showed similar tubular formation as the negative control, with many tubules being short or broken. The 10% FBS hypertrophic groups was similar to the positive control, and thick tubules are indicated with red arrows. Finally, the wnt agonist

group displayed tubular formation between the chondrogenic control and the 10% FBS group.

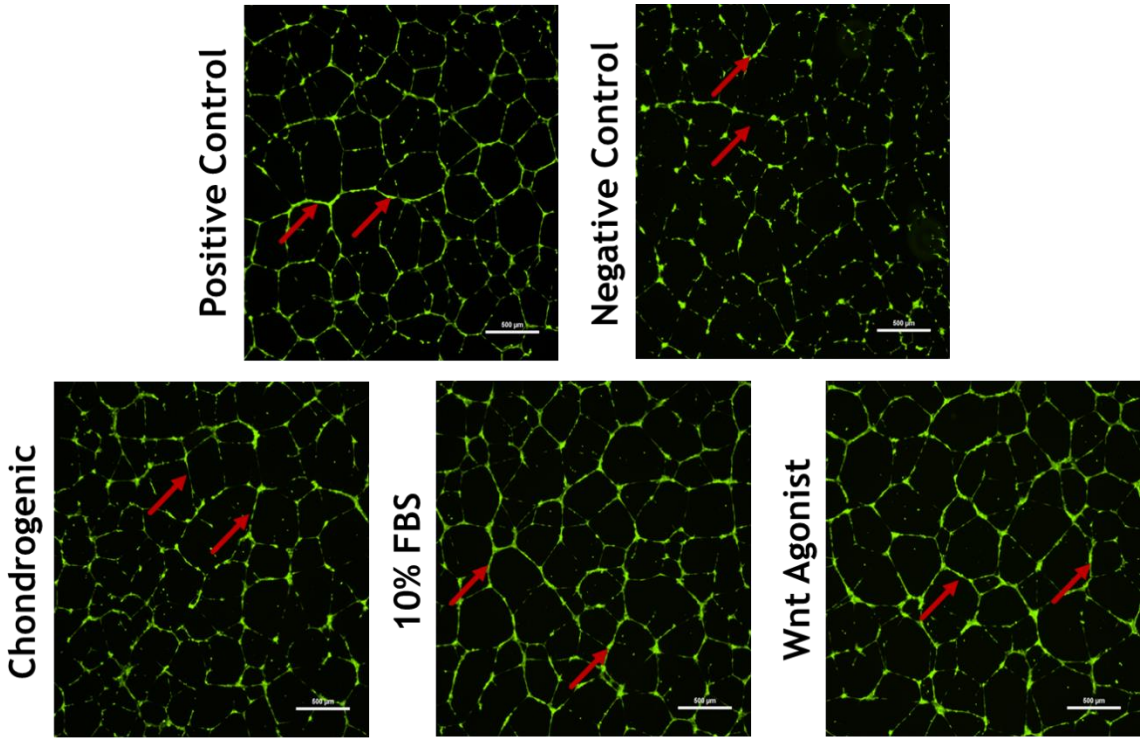

Figure 8: Qualitative images of HUVEC tubular formation

Because it is difficult to assess tubular formation visually, an ImageJ plug in was used to quantify the images. An example of a quantified image is show in Figure 9A. The results from the quantification are shown in Figure 9B. The only significant difference seen here is between the 10% FBS group and the negative control, with the 10% FBS group exhibiting more tubular formation.

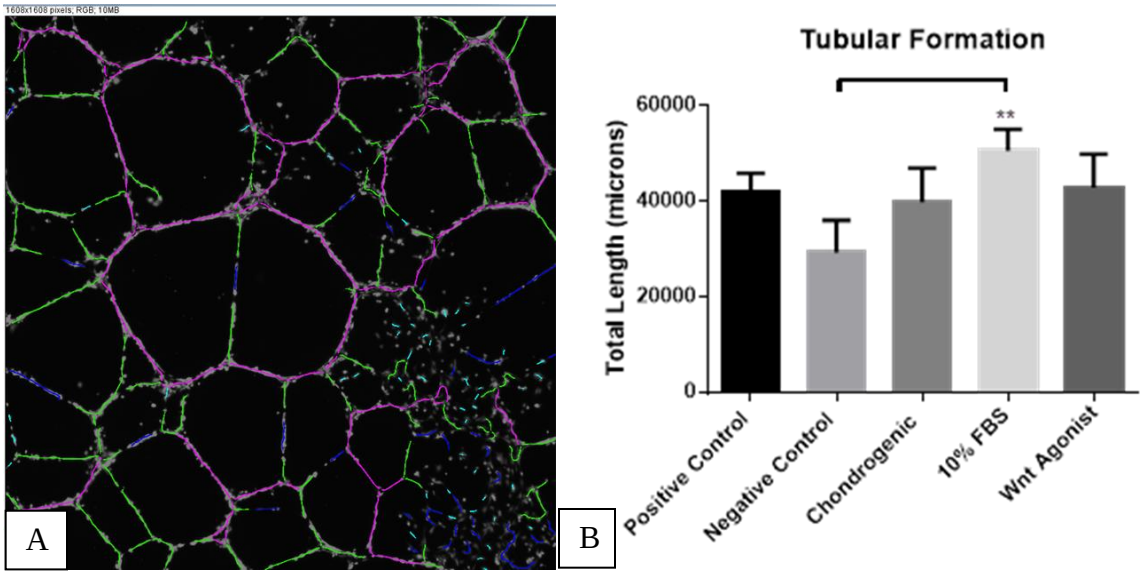

Figure 9: Quanitified HUVEC tubular formation. Bar indicates significane (P < 0.01)

Discussion and Conclusion

Summary

This study sought to characterize hypertrophic differentiation of CEP cells by using two factors shown to induce hypertrophy in OA chondrocytes, 10% FBS and wnt agonist. To characterize differentiation tests were run to examine viability, gene expression of matrix markers, gene expression of hypertrophic markers, and gene expression of neurovascular and pain markers. Downregulations were seen in matrix marker COL2 for hypertrophic groups, and upregulations seen in hypertrophic markers for hypertrophic groups. Neurovascular and pain markers also exhibited upregulations in the hypertrophic groups. Matrix production was assessed through histological analysis and DMMB assay, which both revealed decreased proteoglycan content in hypertrophic groups. To confirm

angiogenic potential of CEP cells, a functional assay of angiogenesis was performed via HUVEC tubular formation, and increased tubular formation was seen in the 10% FBS hypertrophic group.

Discussion based on hypothetical model

Hypertrophy

The first goal of this study was to determine if CEPs could undergo hypertrophic differentiation. To do so, cells were cultured in medium known to induce hypertrophy, and gene expression evaluated. As shown in OA literature, hypertrophic chondrocytes will display upregulations in specific genes, including IHh, MMP13, and Runx2. Our culture model showed significant upregulations of MMP13 and Runx2 in the 10% FBS group. Though not significant, IHh, MMP13 for the wnt agonist group, and Runx2 for the wnt agonist group all displayed upregulations. Sample size for gene expression was between N=3 and N=5, which is relatively small, and with increased sample size groups may become significant.

Additionally, viability of pellets was assessed. The chondrogenic control group showed the most viability, suggesting that the 21-day culture in hypoxia did not affect cell survival. The hypertrophic groups, however, displayed more cell death, especially in the 10% FBS group. Normally this amount of cell death would be concerning. However, during hypertrophic differentiation, chondrocytes eventually undergo apoptosis to allow for matrix calcification. Because only the hypertrophic groups displayed this amount of cell death, it supports CEP hypertrophic differentiation by 10% FBS and wnt agonist.

These results were similar to those seen in the hypertrophic differentiation of OA cells, as described in Pessese *et al* and Enochson *et al*. 10% FBS was able to induce a hypertrophic phenotype in OA chondrocytes, and increases in hypertrophic markers MMP13 and Runx2 were seen (Pesesse et al., 2013). Pesesse *et al* were also able to show the morphology of the cells, and confirm cells were hypertrophic through increased intracellular volume. Because this study utilized high density pellet culture, it is difficult to determine cellular morphology. Enochson *et al* similarly showed that OA chondrocytes could become hypertrophic via a wnt agonist, as confirmed by hypertrophic and matrix markers (SOX9, COL2, MMP13).

Previous work has shown that hypertrophic CEPs can be rescued with ligustrazine, which suppressed TGF-β1 expression (Liu et al., 2016). However, hypertrophic CEPs in Liu *et al* were not fully characterized as hypertrophic, but rather osteogenic, and endochondral ossification was not considered.

Catabolism

After determining CEPs could undergo hypertrophic differentiation, the effect on matrix catabolism by hypertrophic CEPs was explored. To do so, histological staining with picrosirius red and alcian blue was performed and a DMMB assay ran. The chondrogenic control group showed the most proteoglycan content, which was confirmed by detection of GAG chains via the DMMB assay. The least proteoglycan was seen in the 10% FBS group. Again, the wnt agonist group displayed results between the control and 10% FBS groups. The disappearance of

proteoglycan in the hypertrophic groups can be explained either by down regulation of matrix proteins, by upregulation of catabolic enzymes, or by a combination of the two. Due to the significant upregulation of the catabolic gene MMP13, it is likely that catabolism is involved.

In previously described work on OA chondrocytes, similar effects were seen on catabolism and matrix production. Both Pesesse *et al* and Enochson *et al* showed increases in the catabolic gene MMP13. Additionally, Enochson *et al*, through histological analysis, showed that hypertrophic chondrocytes produced less proteoglycan. The difference in their study was that they were able to regain matrix production via GDF5, which reduced MMP13 expression.

Angiogenesis

The CEP is normally only thought to have a nutritional role in the IVD. It has been shown that the CEP calcifies in degeneration, but the effects of this have only been explored with relevance to metabolite transfer. The second aim of this study was to show that hypertrophic CEPs release factors that affect the surrounding environment. First, gene expression of neurovascular and pain markers was assessed. In both hypertrophic groups, upregulation of neurogenic marker, NGF, and angiogenic marker, VEGF were seen. This supports the role of the CEP in influencing the symptomatic IVD, which has nerve and blood vessel ingrowth.

Gene expression is an indication of protein synthesis, but to confirm the angiogenic potential of hypertrophic CEPs, a functional assay of angiogenesis was performed. HUVECs were cultured with conditioned media generated from each condition,

and tubular formation assessed. Increases were seen in the 10% FBS hypertrophic group, further supporting angiogenesis caused by degenerate CEPs.

Pesesse *et al* determined the influence of hypertrophic OA chondrocytes on angiogenesis. To determine angiogenic potential of the hypertrophic chondrocytes, they performed assays to measure adhesion, migration, and invasion of endothelial cells. Similar to their study, we showed that hypertrophic CEPs can influence angiogenesis.

Significance and conclusion

These results suggest that human CEPs can undergo hypertrophic differentiation similar to what is seen in the progression of OA. This study also supports the role of hypertrophic CEPs to enhance angiogenesis and pain in the degenerate IVD. Additionally, this work highlights a unique role for CEP hypertrophy in mechanisms underlying the pathogenesis of disc degeneration and low back pain, and provides a potential target for therapeutics.

Limitations and Future Directions

Collagen type X

Collagen type X (COLX) is a well-known indicator of hypertrophic differentiation in chondrocytes (Pesesse et al., 2013). In the current study, COLX gene expression was determined for 3 samples, but no expression was seen (data not shown). One explanation is the stage of hypertrophic differentiation COLX is expressed. It has been determined that COLX is expressed by early hypertrophic cells (Kozhemyakina, Lassar, & Zelzer, 2015).

Because other factors, such as VEGFA, Runx2, and MMP13 are all upregulated during the late hypertrophic stage, it is likely that COLX expression was upregulated at an earlier timepoint. By the 21-day time point, COLX expression may have disappeared. To determine if protein synthesis occurred, pellets can be examined for COLX via immunohistochemistry.

Chondrocyte de-differentiation

It is known that chondrocytes and chondrocyte-like cells such as the NP dedifferentiate in monolayer expansion. Cells lose their chondrogenic phenotype, shown through decreased collagen type II and aggrecan production, even after 21 days in 3D pellet culture and chondrogenic media (Rosenzweig et al., 2017). Because CEPs were expanded in monolayer, this dedifferentiation may have occurred, affecting the matrix production in this study. While this is an obvious limitation, pellet culture attempts to regain the chondrogenic phenotype of the cells and is a commonly used technique.

Functional assay of angiogenesis

While the tubular formation assay is an indicator of vascularization, it may not be the best model to describe angiogenesis. To fully explore the angiogenic effect of hypertrophic CEP cells, more relevant tests should be completed. Examples of these tests include cellular invasion, migration, and adhesion assays, as described previously (Pesesse et al., 2013).

Future directions

This aims of this study were to examine the effect of hypertrophic differentiation of CEP cells and to confirm using a functional assay of angiogenesis, but future studies to

determine the mechanism are needed. To confirm that IHh is the pathway to hypertrophy for CEPs, we will perform receptor blocking studies and RNA interference of IHh. Receptor blocking will be done via cyclopamine, an antagonist for IHh (Chen, Taipale, Cooper, & Beachy, 2002). Small (or short) interfering RNA, also known as siRNA, will be used for temporary gene knockdown via messenger RNA degradation. If these methods are successful in abolishing hypertrophic differentiation, therapeutic agents that target IHh in the IVD can be explored.

Future studies can also be used to determine the effects of hypertrophic CEPS on NP and AF cells. To do so, conditioned media generated from the hypertrophic CEPs can be introduced to the NP and AF cells. The gene expression and matrix production of these cells can be determined like was described in this study. Results will elucidate the role of the CEP-IVD interactions. Additionally, this may suggest that the CEP is more than a nutritional pathway for the IVD, but rather plays a role in the biochemical microenvironment of the IVD.

References

Adams, M. A., & Roughley, P. J. (2006). What is Intervertebral Disc Degeneration, and What Causes It? *Spine*, *31*(18), 2151–2161. https://doi.org/10.1097/01.brs.0000231761.73859.2c

Andersson, G. (1999). Epidemiological features of chronic low-back pain. *Lancet*, *354*, 581–5.

Borczuk, A. C., Gorenstein, L., Walter, K. L., Assaad, A. A., Wang, L., & Powell, C. A. (2003). Non-Small-Cell Lung Cancer Molecular Signatures Recapitulate Lung Developmental Pathways. *American Journal of Pathology*, *163*(5), 1949–1960. https://doi.org/10.1016/S0002-9440(10)63553-5

Chen, J. K., Taipale, J., Cooper, M. K., & Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened, (410), 2743–2748. https://doi.org/10.1101/gad.1025302.GENES

de Schepper, E. I. T., Damen, J., van Meurs, J. B. J., Ginai, A. Z., Popham, M., Hofman, A., … Bierma-Zeinstra, S. M. (2010). The association between lumbar disc degeneration and low back pain: the influence of age, gender, and individual radiographic features. *Spine*, *35*(5), 531–536. https://doi.org/10.1097/BRS.0b013e3181aa5b33

DeLucca, J. F., Cortes, D. H., Jacobs, N. T., Vresilovic, E. J., Duncan, R. L., & Elliott, D. M. (2016). Human cartilage endplate permeability varies with degeneration and intervertebral disc site. *Journal of Biomechanics*, *49*(4), 550–557.

https://doi.org/10.1016/j.jbiomech.2016.01.007

Donovan, D., Brown, N. J., Bishop, E. T., & Lewis, C. E. (2001). Comparison of three in vitro human “angiogenesis” assays with capillaries formed in vivo. *Angiogenesis*, *4*(2), 113–121. https://doi.org/10.1023/A:1012218401036

Dudli, S., Fields, A. J., Samartzis, D., Karppinen, J., & Lotz, J. C. (2016). Pathobiology of Modic changes. *European Spine Journal*, *25*(11), 3723–3734. https://doi.org/10.1007/s00586-016-4459-7

Enochson, L., Stenberg, J., Brittberg, M., & Lindahl, A. (2014). GDF5 reduces MMP13 expression in human chondrocytes via DKK1 mediated canonical Wnt signaling inhibition. *Osteoarthritis and Cartilage*, *22*(4), 566–577. https://doi.org/10.1016/j.joca.2014.02.004

Freemont, A. J. (2009). The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. *Rheumatology*, *48*(1), 5–10. https://doi.org/10.1093/rheumatology/ken396

Goldring, M. B. (2012). Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Therapeutic Advances in Musculoskeletal Disease*, *4*(4), 269–85. https://doi.org/10.1177/1759720X12448454

Kozhemyakina, E., Lassar, A. B., & Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development*, *142*(5), 817–831. https://doi.org/10.1242/dev.105536

Lawrence, R. C. (2008). NIH Public Access. *Arthritis Rheum*, *58*(1), 26–35.

https://doi.org/10.1002/art.23176.Estimates

Liu, S., Zhao, B., Shi, H., Liang, Q., Fu, Y., Yang, Z., … Bian, Q. (2016). Ligustrazine Inhibits Cartilage Endplate Hypertrophy via Suppression of TGF-?? 1. *Evidence-Based Complementary and Alternative Medicine*, *2016*. https://doi.org/10.1155/2016/1042489

Luo, X.-W., Liu, K., Chen, Z., Zhao, M., Han, X.-W., Bai, Y.-G., & Feng, G. (2016). Adenovirus-mediated GDF-5 promotes the extracellular matrix expression in degenerative nucleus pulposus cells. *Journal of Zhejiang University. Science. B*, *17*(1), 30–42. https://doi.org/10.1631/jzus.B1500182

Maetzel, A., & Li, L. (2002). The economic burden of low back pain: a review of studies published between 1996 and 2001. *Best Practice & Research in Clinical Rheumatology*, *16*(1), 23–30. https://doi.org/10.1053/berh.2002.0204

Mannion, A. F., Leivseth, G., Brox, J.-I., Fritzell, P., Hägg, O., & Fairbank, J. C. T. (2014). ISSLS Prize Winner. *Spine*, *39*(17), 1373–1383. https://doi.org/10.1097/BRS.0000000000000437

Nishimura, R., Wakabayashi, M., Hata, K., Matsubara, T., Honma, S., Wakisaka, S., … Yoneda, T. (2012). Osterix regulates calcification and degradation of chondrogenic matrices through matrix metalloproteinase 13 (MMP13) expression in association with transcription factor Runx2 during endochondral ossification. *Journal of Biological Chemistry*, *287*(40), 33179–33190. https://doi.org/10.1074/jbc.M111.337063

Pesesse, L., Sanchez, C., Delcour, J. P., Bellahcène, A., Baudouin, C., Msika, P., & Henrotin, Y. (2013). Consequences of chondrocyte hypertrophy on osteoarthritic cartilage: Potential effect on angiogenesis. *Osteoarthritis and Cartilage*, *21*(12), 1913–1923. https://doi.org/10.1016/j.joca.2013.08.018

Purmessur, D., Schek, R. M., Abbott, R. D., Ballif, B. A., Godburn, K. E., & Iatridis, J. C. (2011). Notochordal conditioned media from tissue increases proteoglycan accumulation and promotes a healthy nucleus pulposus phenotype in human mesenchymal stem cells. *Arthritis Research & Therapy*, *13*(3), R81. https://doi.org/10.1186/ar3344

Rosenzweig, D. H., Gravel, J. T., Bisson, D., Ouellet, J. A., Weber, M. H., & Haglund, L. (2017). Comparative Analysis in Continuous Expansion of Bovine and Human Nucleus Pulposus Cells for Tissue Repair Applications. *European Cells and Materials*, *33*, 240–251. https://doi.org/10.22203/eCM.v033a18

Selman, M., Pardo, A., & Kaminski, N. (2008). Idiopathic pulmonary fibrosis: Aberrant recapitulation of developmental programs? *PLoS Medicine*, *5*(3), 0373–0380. https://doi.org/10.1371/journal.pmed.0050062

Smith, L. J., Nerurkar, N. L., Choi, K.-S., Harfe, B. D., & Elliott, D. M. (2011). Degeneration and regeneration of the intervertebral disc: lessons from development. *Disease Models & Mechanisms*, *4*(1), 31–41. https://doi.org/10.1242/dmm.006403

Walter, B. A., Korecki, C. L., Purmessur, D., Roughley, P. J., Michalek, A. J., & Iatridis, J. C. (2011). Complex loading affects intervertebral disc mechanics and biology. *Osteoarthritis and Cartilage*, *19*(8), 1011–1018.

https://doi.org/10.1016/j.joca.2011.04.005